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(54) Title: COMPOSITIONS AND METHODS FOR DETECTION OF GENOMIC IMPRINTING DISORDERS

(57) Abstract

The present invention relates to methods and compositions for identifying genetic abnormalities associated with genomic imprinting disorders. In certain embodiments, the method of the present invention involves digestion of sample DNA with methylation–sensitive restriction enzymes followed by amplification or by ligation to anchor–primers and subsequent amplification. The present invention facilitates rapid and thorough diagnosis of the major genotypes responsible for genomic imprinting syndromes.

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COMPOSITIONS AND METHODS FOR DETECTION OF GENOMIC IMPRINTING DISORDERS

FIELD OF THE INVENTION

The present invention relates to methods and compositions for identifying genetic abnormalities associated with genomic imprinting disorders.

BACKGROUND OF THE INVENTION

Genomic Imprinting

Genomic imprinting has recently gained great attention, progressing from an interesting embryological curiosity into an established field with broad repercussions on human development and a variety of disease states. Defects in genomic imprinting have been associated with severe developmental disorders including Prader-Willi syndrome, Angelman syndrome, Beckwith-Wiedemann syndrome, and several types of tumors including Wilms' tumor, neuroblastoma, rhabdomyosarcoma, and lung cancer (*see e.g., Genomic Imprinting, Causes and Consequences*, Cambridge University Press [1995]).

Genomic imprinting results from the preferential expression of one parental allele over the other. Experimentation in the 1980s demonstrated that the maternally and paternally contributed chromosomes of an embryo are not functionally identical, and that both are necessary for normal development (*see e.g.*, McGrath and Solter, Cell 37: 179 [1984]; and Cattanach and Kirk, Nature 315: 496 [1985]). In a classical genomic imprinting model, one of the chromosomes contains genes that are transcriptionally inactivated, leaving its complement to contribute all or most of the genetic information. Disruption of the active allele causes loss of gene product and may result in developmental abnormalities.

Prader-Willi and Angelman syndromes

Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are distinct neurobehavioral disorders, each affecting approximately 1/10,000 to 1/20,000 children (Holm *et al.*, Pediatrics 91: 398 [1993]). However, because of difficulty in clinical detection and limitations in current screening tests, the true incidence is unknown (Williams *et al.*, Curr. Prob. Pediatrics, Aug: 216 [1995]). Both syndromes illustrate the phenomenon of genomic imprinting, in which there is abnormal expression of genes that normally function only when inherited from the father or from the mother (Horsthemke *et al.*, *Genomic Imprinting, Causes and Consequences*, Cambridge University Press, 295 [1995]).

Prader-Willi Syndrome

Prader-Willi syndrome (PWS) is characterized by neonatal hypotonia, hypogonadism, mild to moderate mental retardation, learning disabilities, obsessive-compulsive disorder, short stature, and morbid obesity due to hyperphagia beyond infancy (Dykens *et al.*, J. Child Pyschol. Psychiat. 37: 995 [1996]). PWS is linked to genes on chromosome 15, with several loci subject to allele-specific gene expression and DNA methylation (Ledbetter *et al.*, New Engl. J. Med. 304: 325 [1981]) . The active genes are expressed from the paternally contributed chromosome which is unmethylated in important transcriptional regulatory regions. The maternal chromosome is heavily methylated and contains inactive copies of the genes (Horsthemke *et al.*, *supra*). Several genes, when not expressed, may be responsible for PWS. Small nuclear ribonucleoprotein particle associated polypeptide SmN (SNRPN) was the first gene encoding a defined protein that was mapped to the imprinted region on chromosome 15 (Özcelik *et al.*, Nature Genet. 2: 265 [1991]). The SNRPN gene product participates in RNA splicing in the brain. Two other genes, IPW and ZNF127, and two transcripts, PAR-5 and PAR-1, are expressed in a parent-specific manner within the imprinted region of chromosome 15, but have not been well characterized. A schematic figure describing the imprinted region of chromosome 15

is presented in Figure 1 and the DNA sequence of the SNRPN CpG island is presented in Figure 2 (SEQ ID NO: 1). The imprinted region lies 3' of the chromosome 15 centromere (cen) and spans 15q11-13. The jagged lines in Figure 1 represent breakage points between the centromere and telomere (tel) that are commonly associated with deletions in the imprinting region.

Approximately 70-75% of PWS patients have a large cytogenetic deletion from q11 to q13 on the paternal copy of chromosome 15 (Nicholls *et al.*, Am. J. Med. Genet. 33: 66 [1989]). Twenty to twenty-five percent of PWS patients have maternal uniparental disomy (UPD) of chromosome 15, in which both copies of chromosome 15 have been inherited from the mother (Mascari *et al.*, New Eng. J. Med. 326: 1599 [1992]). Two to five percent of PWS patients have a mutation in the imprinting process, in which DNA methylation and imprinted gene expression are abnormal throughout 15q11-q13 (Reis *et al.*, Am. J. Hum. Genet. 54: 741 [1994]; and Saitoh *et al.*, Am. J. Med. Genet. 68: 195 [1997]), while less than 0.5% of PWS cases are caused by a balanced, paternally derived chromosome 15 rearrangement (Sun *et al.*, Hum. Mol. Genet. 5: 517 [1996]; and Schulze *et al.*, Nat. Genet 12, 452 [1996]).

Angelman Syndrome

Angelman syndrome (AS) is characterized by severe mental retardation, lack of speech, epilepsy, easily evoked laughter, hyperactivity, and ataxic gait (Williams *et al.*, Curr. Prob. Pediatr. 25: 213 [1995]). AS is linked to the same imprinted region on chromosome 15 as PWS, although the gene(s) associated with the disease has not been clearly identified. Unlike PWS, the active gene(s) is expressed from the maternally contributed chromosome. The paternal chromosome contains an inactive copy of the gene(s).

Similar to PWS, 70-75% of AS patients have a large cytogenetic deletion of the chromosome 15q11-q13 region (Williams *et al.*, Curr. Probl. Pediatr. 25: 216 [1995]), but with the deletion always on the maternally derived chromosome. One to two percent of AS patients have paternal UPD of chromosome 15, with both copies of

the chromosome inherited from the father (Williams *et al.*, [1995], *supra*), while 5% of AS patients have a mutation in the imprinting process (Saitoh *et al.*, Proc. Natl. Acad. Sci. USA 93: 7811 [1996]). The remaining AS patients (approximately 20%) are thought to have a mutation in the AS gene(s) (Kishino *et al.*, Nat. Genet. 15, 70 [1997]). A schematic diagram of the molecular genotypes of Angelman and Prader-Willi patients is presented in Figure 3 where paternal (P) and maternal (M) alleles are shown with gaps representing deletions, dotted portions representing imprinting mutations, and diagonally shaded portions representing translocated sections of the chromosome.

Diagnosis

Although several methods are currently used to diagnose PWS and AS, all of these techniques suffer from significant shortcomings. None of the methods currently available allow for quick diagnosis and permit the detection of each of the major genotypes of PWS and AS. These drawbacks prevent the rapid, simple, and complete detection of the syndromes and thus, preclude their use in population-based screening of neonates, infants, and children for the syndromes. Such a method is needed because current phenotypic diagnostic criteria are subtle, nonspecific, and can change with age, leading to underdiagnosis in younger patients and overdiagnosis in obese, retarded, older patients (Holm *et al.*, Pediatrics 91: 398 [1993]).

The methods currently used to diagnose PWS and AS include standard cytogenetics, fluorescence in situ hybridization (FISH) (Cassidy *et al.*, Am. J. Hum. Genet. 58: 1085 [1996]), microsatellite analysis by polymerase chain reaction (PCR) (Mutirangura *et al.*, Hum. Mol. Genet. 2: 143 [1993]), RNA analysis (Glenn *et al.*, Hum. Mol. Genet. 2: 2001 [1993]; and Wevrick and Francke, Lancet 348: 1068 [1996]), and Southern blot tests including restriction fragment length polymorphism (RFLP) (Nicholls *et al.*, Am J. Med Genet. 33: 66 [1989]), DNA dosage (Tantravahi *et al.*, Am. J. Med. Genet. 33, 78 [1989]), and DNA methylation (*see eg.*, Driscoll *et al.*, Genomics 13, 917 [1992]).

These genetics-based testing methods have significant drawbacks. For example, standard cytogenetics and FISH can only detect deletions and translocations. In addition, standard cytogenetic methods are known to have problems associated with false-negative and false-positive results and cannot detect small deletions or translocations. Microsatellite analysis only detects uniparental disomy, and deletions if informative microsatellites are within the deleted region, and requires DNA from both parents, which is not always available. RNA analysis uses RNA, which is difficult to work with in a diagnostic setting and can only identify PWS cases. The Southern blot tests are time consuming. Furthermore, RFLP methods only detect deletions and uniparental disomy and are often uninformative and DNA dosage methods only detect deletions and suffer from unreliable interpretations. DNA methylation tests can detect deletions, uniparental disomy, and imprinting mutations but require large amounts of DNA and several days for analysis. Thus, methods and compositions suitable for routine, rapid, and thorough diagnosis of genomic imprinting diseases such as PWS and AS are sorely needed.

SUMMARY OF THE INVENTION

The present invention relates to methods and compositions for identifying genetic abnormalities associated with genomic imprinting disorders.

In one embodiment, the present invention provides a method for diagnosing genomic imprinting disorders comprising: providing a biological sample suspected of containing genomically imprinted DNA, two or more nucleotide primers that are complementary to a portion of human chromosome 15, at least one methylation-sensitive restriction enzyme, and DNA polymerase; isolating the DNA from a biological sample; digesting the DNA with a methylation-sensitive restriction enzyme to create a restriction product; exposing the primers to the restriction product; and amplifying the restriction product to produce amplification products.

In one alternative embodiment, the method of the present invention further comprises the step of comparing the amplification products to at least one control sample. The controls can consist of, but are not limited to, DNA samples from

patients with known genotypes or samples known to produce, or not to produce, amplification products.

In a preferred embodiment of the method of the present invention, Angelman syndrome is the genomic imprinting disorder that is diagnosed.

In an alternate embodiment of the method of the present invention, the biological sample is from a patient suspected of having DNA damage. In a preferred embodiment the DNA damage is caused by a mutagen selected from the group consisting of chemical and radiation mutagens.

In a preferred embodiment, the amplifying comprises the polymerase chain reaction. However, it is not intended that amplification be limited to PCR, as all amplification techniques are contemplated.

In yet another preferred embodiment, the polymerase is thermostable. Although it is not intended that the invention be limited to a particular thermostable polymerase, it is contemplated that polymerases including, but not limited to, *Taq*, Vent, and Pfu be used.

In yet another preferred embodiment, the portion of chromosome 15 comprises the sequence between 15q11-15q13.

In a preferred embodiment, the primers flank the SNRPN gene. In a particularly preferred embodiment, the primers are selected from the group consisting of SEQ ID NO: 12 and SEQ ID NO: 13. However, it is not intended that the invention be limited to these primers, as any primers that produce amplification products from the sample are contemplated.

In a preferred embodiment, the methylation-sensitive restriction enzyme is selected from the group consisting of *NotI* and *HhaI*. However, it is not intended that the invention be limited to these specific methylation-sensitive restriction enzymes.

In another preferred embodiment of the method of the present invention, the DNA is genomic DNA.

In one embodiment, the present invention provides a nucleotide sequence that is used in the amplification reactions comprising at least a portion of the nucleic acid sequence complementary to SEQ ID NO: 1, or variants thereof. In a preferred

embodiment, the portion comprises nucleic acid sequence selected from the group consisting of SEQ ID NO: 12 and SEQ ID NO: 13.

The present invention also provides methods for diagnosing genomic imprinting disorders comprising: providing a biological sample suspected of containing genomically imprinted DNA, at least two nucleotide primers, at least one methylation-sensitive restriction enzyme, a means of ligation, one or more anchor primers, and DNA polymerase; isolating the DNA from a biological sample; digesting the DNA with a methylation-sensitive restriction enzyme to create a restriction product; ligating the anchor primers to the restriction product using the means of ligation; exposing the primers to the restriction product; and amplifying the restriction product to produce amplifications products.

In an alternative embodiment, the method of the present invention further comprises the step of comparing the amplification products to at least one control sample. The controls can consist of, but are not limited to, DNA samples from patients with known genotypes or samples known to produce, or not to produce, amplification products.

In a preferred embodiment of the method of the present invention, Prader-Willi syndrome is the genomic imprinting disorder that is diagnosed.

In an alternate embodiment of the method of the present invention, the biological sample is from a patient suspected of having DNA damage. In a preferred embodiment the DNA damage is caused by a mutagen selected from the group consisting of chemical and radiation mutagens.

In a preferred embodiment, the amplifying comprises the polymerase chain reaction. However, it is not intended that amplification be limited to PCR, as all amplification techniques are contemplated.

In yet another preferred embodiment, the polymerase is thermostable. Although it is not intended that the invention be limited to a particular thermostable polymerase, it is contemplated that polymerases including, but not limited to, *Taq*, Vent, and Pfu be used.

In yet another preferred embodiment, the nucleotide primers are complementary to a portion of human chromosome 15. In a particularly preferred embodiment, the portion of chromosome 15 comprises the sequence between 15q11-15q13.

In a preferred embodiment, the primers flank the SNRPN gene. In a particularly preferred embodiment, the primers are selected from the group consisting of SEQ ID NO: 12 and SEQ ID NO: 13. However, it is not intended that the invention be limited to these primers, as any primers that produce amplification products from the sample are contemplated.

In a preferred embodiment, the methylation-sensitive restriction enzyme is selected from the group consisting of *NotI* and *HhaI*. However, it is not intended that the invention be limited to these specific methylation-sensitive restriction enzymes.

In another preferred embodiment of the method of the present invention, the DNA is genomic DNA.

DESCRIPTION OF THE FIGURES

Figure 1 is a schematic diagram of the genomically imprinted region of human chromosome 15.

Figure 2 shows the DNA sequence for the SNRPN CpG island (SEQ ID NO: 1).

Figure 3 is a schematic diagram of the molecular genotypes of Angelman and Prader-Willi patients.

Figure 4 is a schematic diagram showing the strategy of Methylation PCR. Panel (a) shows the sample prior to restriction digestion. Panel (b) shows the sample following restriction digestion.

Figure 5 is a schematic diagram showing the strategy of Methylation-Anchor PCR. Panel (a) shows the sample prior to restriction digestion. Panel (b) shows the sample following restriction digestion.

Figure 6 is a photograph of an agarose gel, showing the results of Methylation-Anchor PCR analysis of blood samples from Angelman and Prader Willi patients.

Figure 7 is a photograph of an agarose gel, showing the results of Methylation -PCR analysis of blood samples from Angelman and Prader Willi patients.

Figure 8 is a photograph of an agarose gel, showing the results of Methylation-Anchor PCR analysis of dried blood spots from Angelman and Prader Willi patients.

Figure 9 is a photograph of an agarose gel, showing the results of Methylation PCR analysis of dried blood spots from Angelman and Prader Willi patients.

DESCRIPTION OF THE INVENTION

The present invention relates to methods and compositions for identifying genetic abnormalities associated with genomic imprinting disorders. It provides methods and compositions for the rapid comparison in methylation patterns in DNA samples. In particular, the present invention makes use of methylation-sensitive restriction enzymes and DNA amplification to assay the abnormal loss or acquisition of methylated DNA in chromosomes. In preferred embodiments, the present invention provides compositions and methods for the rapid detection of DNA deletions, uniparental disomy, and genomic imprinting mutations associated with Prader-Willi syndrome, Angelman syndrome, and other genomic imprinting disorders. The present invention has numerous advantages and overcomes the drawbacks of currently available diagnostic methods as it allows rapid and simultaneous diagnosis of each of the major genotypic forms of Angelman and Prader-Willi syndromes, and requires only a small amount of test sample (*e.g.*, tissue or DNA). Additionally, the present invention distinguishes between AS and PWS and can diagnose both syndromes simultaneously from a single experimental sample. This improved diagnostic test is optimal for initial diagnosis of AS and PWS, confirmation of a suspected clinical diagnosis, and application to population-based screening for these syndromes.

It is contemplated that the methods and compositions of the presently claimed invention will find widespread use for rapid diagnosis of many of the syndromes related to genomic imprinting. This is of particular importance in view of the increasing recognition of genomic imprinting as having an important role in various disease states. Several types of genetic changes are known to disrupt the normal

expression of imprinted genes and lead to developmental disorders. Large deletions on the chromosome containing the active allele can disrupt or delete imprinted genes, causing loss of function. Uniparental disomy (UPD), a duplication of one allele with loss of the other, will result in the loss of function if the inactive allele is duplicated. Mutations within important imprinting regions can disrupt the active copy by altering its chromatin structure or methylation pattern and thus its transcription. Chromosome rearrangements or mutations within the genes can also lead to loss of function on the active allele. These chromosome alterations in genomically imprinted regions have been linked to several forms of embryonic tumors including Wilms' tumor (Wilkins, Lancet i: 329 [1988]), neuroblastoma (Caron *et al.*, Nature Genet. 4: 187 [1990]), rhabdomyosarcoma (Scrable *et al.*, Proc. Natl. Acad. Sci. USA 86: 7480 [1989]) and lung cancer (Suzuki *et al.*, Nature Genet. 6: 332 [1994]). Genomic imprinting defects have also been found to cause Beckwith-Wiedemann syndrome (Weksberg and Squire, *In Genomic Imprinting, Causes and Consequences*, Cambridge University Press, 237 [1995]). To the extent that these, and yet undiscovered, conditions result from alterations in methylated DNA between active and inactive parental chromosomes, the disclosed invention provides effective new compositions and methods for prompt and accurate diagnosis by simultaneously detecting deletions, imprinting mutations, and uniparental disomy on both alleles.

It is also contemplated that the presently claimed invention will be applied to epidemiological studies of populations exposed to potential chemical or radiation mutagens. Studies have demonstrated a correlation between parental exposure to chemical mutagens and the occurrence of Prader-Willi syndrome in children (Cassidy *et al.*, Am. J. Hum. Genet. 44: 806 [1989]). The availability of samples from these studies, as well as research involving DNA damage from radiation mutagens (Neel, Am. J. Hum. Genet. 57: 1263 [1995]; and Dubrova *et al.*, Nature 380: 683 [1996]) permits screening of the samples by the presently claimed invention for genomic imprinting syndromes.

There are several common features present in all of the imprinted genes that have been studied thus far. First, the whole genome is not imprinted, but only specific genes within specific chromosomal regions on certain chromosomes (Cattanach *et al.*, *In Genomic Imprinting, Causes and Consequences*, Cambridge University Press, 327 [1995]). Also, the suppressed allele is almost completely inactivated, with inactivation occurring at the transcriptional level. Transcriptional inactivation is caused by allele-specific chromatin configurations and/or DNA methylation.

DNA is subject to postsynthetic modification by addition of methyl groups ($-CH_3$). Many organisms possess methylated DNA, with methylation occurring at the C5 of cytosine or the N6 of adenosine residues. In animals, methylation occurs on cytosine residues that are located directly 5' of guanosine residues, with most of the CG dinucleotides in mammalian genomes being methylated. Evidence suggests that methylation in eukaryotes contributes to the transcriptional regulation of gene expression. Methylated CG dinucleotides can directly regulate transcription by interacting with transcription factors or can indirectly regulate transcription by modifying chromatin structure and altering the access of promoters to transcription factors.

Two percent of the mammalian genome contains short regions of CG dinucleotides that are free of methylation (Bird, Trends Genet. 3: 342 [1987]). These "CpG islands" are unusually CG rich and are common in housekeeping genes and genes that are active only in specific tissues. In certain chromosomal regions, the sequences containing CpG islands are unmethylated on one of the alleles and methylated on the other, a pattern that is characteristic of many genomically imprinted genes.

In one embodiment of the method of the present invention, methylation-sensitive restriction enzyme digestion and DNA amplification are used to diagnose syndromes associated with genomically imprinted regions of chromosomes. Many genomically imprinted regions are methylated on one allele and unmethylated on the other. One copy contains active genes, the other is inactive. Deletions and

mutations in the active copy or duplication of the inactive copy causes complete loss of gene function and the associated syndromes.

Using a combination of methylation-PCR and methylation-anchor PCR, deletions, duplications, and imprinting mutations can be detected in both alleles. Methylation-PCR detects the methylated allele from normal individuals (class 1), both alleles from patients with uniparental disomy of the methylated allele (class 2), both alleles from patients with imprinting mutations that result in the methylation of the normally unmethylated allele (class 3), and the methylated allele from patients with deletions in the unmethylated allele (class 4). Methylation-PCR does not detect either allele from patients with uniparental disomy of the unmethylated allele (class 5), from patients with deletions in the methylated allele (class 6), or from patients with imprinting mutations that cause the normally methylated allele to be unmethylated (class 7).

Methylation-anchor PCR detects the unmethylated allele from normal individuals (class 1), both alleles from patients with uniparental disomy of the unmethylated allele (class 5), the unmethylated allele from patients with deletions in the methylated allele (class 6), and both alleles from patients with imprinting mutations that cause the normally methylated allele to be unmethylated (class 7). Methylation-anchor PCR does not detect either allele from patients with uniparental disomy of the methylated allele (class 2), from patients with imprinting mutations that result in the methylation of the normally unmethylated allele (class 3), and from patients with deletions in the unmethylated allele (class 4). The combination of the two methods distinguishes each of the genotypic classes of patients.

For both techniques, DNA must first be purified from a biological sample. Preferred embodiments employ peripheral blood leukocytes as the sample source. Blood is easily obtained from patients and is usually already available from other diagnostic testing. It is not intended that the invention be limited to the use of blood, as tissues and body fluids can be used as samples for the DNA source. Also, the samples need not be fresh. For example, dried blood has been successfully used. Cell cultures are also an acceptable sample source. It is contemplated that any tissue with

sufficiently intact genomic DNA can be used. It is further contemplated that purified DNA, whether fresh, frozen, or stored, can be analyzed using the methods and compositions of the present invention.

Methylation-PCR

In methylation PCR, DNA purified from the sample is digested to completion with methylation-sensitive restriction enzyme. Any methylation-sensitive restriction enzyme that cuts at least once within the imprinted region can be used. In some preferred embodiments, the enzyme cuts once within the imprinted region. In an alternative preferred embodiment, the enzyme cuts near or within a gene associated with the syndrome to be tested for. A schematic summary of methylation PCR is presented in Figure 4. In this Figure, "A" and "B" indicate the directions of amplifications of the PCR primers, "Hh" and "N" indicate restriction endonuclease digestion sites, the diagonally-shaded box represents exon 1 of SNRPN, section (a) represents the region prior to restriction digestion, and section (b) shows the methylated and unmethylated alleles following restriction digestion.

In one embodiment, the methylation-sensitive restriction enzyme digests the unmethylated allele from class 1 and class 6 genotype patients and both unmethylated alleles from class 5 and class 7 genotype patients.

Following digestion to completion, DNA amplification with primers designed to flank the restriction site is conducted. Any primer pair that flanks the restriction site can be used. In a preferred embodiment, the primers flank the gene or genes associated with the disease syndrome tested for, and anneal far enough from the restriction site to generate fragments that are readily observable after analysis with agarose gel electrophoresis and ethidium bromide staining. Also, in preferred embodiments, the primers are designed to anneal to a portion of the chromosome that is not rich in guanine and cytosine residues. In one preferred embodiment, DNA amplification is carried out by PCR, however it is not intended that the invention be limited to any particular DNA amplification technique.

DNA digested by the methylation-sensitive restriction enzyme is not amplified because the primer pair does not span a continuous region. DNA from patients with deletions in the methylated allele (class 6) will not amplify because they are missing the primer annealing sites. Therefore, class 5, class 6, and class 7 genotype patients will not have amplification products because both alleles are either digested and/or incapable of amplification. Amplification products will be produced from class 1, class 2, class 3, and class 4 genotype patients because at least one of the alleles remains uncut and is amplified. Control DNA can be amplified in a separate reaction to test the performance of the primers and PCR reagents.

The absence of an amplification product is diagnostic of deletions or imprinting mutations on the normally methylated allele or uniparental disomy of the unmethylated allele. The amplification products can be compared to control samples to diagnose these genotypes, however this comparison is not required. In a preferred embodiment the samples are also diagnosed with methylation-anchor PCR and compared, however this is not required. In preferred embodiments, the amplification products are analyzed by agarose gel electrophoresis followed by ethidium bromide staining. Control samples from each of the genotypic classes can be run on the gel to compare banding patterns with the experimental sample. Lack of banding indicates a genetic abnormality in the methylated allele. It is not intended that the invention be limited to any particular DNA detection technique. For example, the amplification reaction can be performed with radionucleotide substrates and assayed by gel electrophoresis analysis followed by autoradiography or other emission-detection techniques. Alternately, systems such as the Sunrise Amplification System (Oncor) may be used to allow immediate detection of fragments following amplification. Indeed, it is contemplated that any method capable of detecting DNA will find use with the present invention.

Methylation-Anchor PCR

In methylation-anchor PCR, genomic DNA is digested to completion with methylation-sensitive restriction enzymes as described above for methylation PCR.

The restriction enzyme used in this method must produce a single-stranded overhang sufficient for subsequent hybridization and ligation of anchor primers. The methylation-sensitive restriction enzyme digests the unmethylated allele from class 1 and class 6 genotype patients and both unmethylated alleles from class 5 and class 7 genotype patients. Neither allele is digested in class 2, class 3, or class 4 genotype patients. A schematic summary of methylation-anchor PCR is presented in Figure 5. In this Figure, "A" and "B" indicate the directions of amplifications of the PCR primers, "Hh" and "N" indicate restriction endonuclease digestion sites, "AP" indicates the direction of amplification of the PCR anchor primer, the diagonally-shaded box represents exon 1 of SNRPN, section (a) represents the region prior to restriction digestion, and section (b) shows the methylated and unmethylated alleles following restriction digestion.

After digestion, the DNA is ligated to anchor primers. Anchor primers are designed to anneal to the single-stranded overhang created by the methylation-sensitive enzyme. The anchor primers only anneal and ligate to samples that were digested in the prior step. Thus, only DNA from unmethylated chromosomes acquire the anchor primer. Following ligation, the DNA is amplified using a primer that anneals to the anchor, along with either of the flanking primers used in the methylation PCR method or a new primer designed in a similar fashion as the flanking primers. In the preferred embodiment, the primers are designed to produce amplification products that are detectable using agarose gel electrophoresis and ethidium bromide staining. DNA from class 1, class, 5, class 6, and class 7 genotype patients produce amplification products. The amplification products are analyzed as describe for methylation PCR. Absence of a fragment is diagnostic for genomic imprinting defects in the unmethylated allele.

In preferred embodiments, both methylation PCR and methylation-anchor PCR methods are conducted on each sample simultaneously, as this facilitates prompt diagnosis of genetic aberrations on both alleles. In preferred embodiments, the primers and restriction sites are designed to create amplification products of different sizes in the methylation PCR and methylation-anchor PCR reactions. Although not required,

control samples from each of the genotypic classes can be added for comparison to the experimental samples.

In one embodiment, methylation PCR and methylation-anchor PCR can diagnose the deletion, uniparental disomy, and imprinting mutation forms of Angelman and Prader-Willi syndromes. Importantly, these methods and compositions find use in general screening programs to detect and diagnose genomic imprinting syndromes. In a preferred embodiment, *NotI* or *HhaI* restriction enzymes (*i.e.*, methylation-sensitive restriction enzymes) are used to digest the genomic DNA. A *NotI* site lies within the first exon of the SNRPN gene and a *HhaI* site lies within the SNRPN promoter. PCR primers can be designed to span methylation sensitive restriction enzyme *NotI* and *HhaI* sites in the SNRPN CpG island, which is completely methylated on the maternally derived chromosome while completely unmethylated on the paternally derived one. In a preferred embodiment, primers corresponding to SEQ ID NO: 12 and SEQ ID NO: 13 are used in the amplification reaction. However, it is not intended that the invention be limited to the use of these specific restriction enzymes or primers. Any methylation-sensitive restriction enzyme that digests at least once within the imprinted region of chromosome 15 can be used. Any primer pair that flanks the chosen restriction site can be used.

When genomic DNA is digested, DNA amplification with these primers results in amplification of only the methylated, normally maternally-derived allele. Ligation of an anchor primer to digested genomic DNA allows ligation-mediated DNA amplification of only the unmethylated, normally paternally derived allele by using the anchor primer and either of the SNRPN CpG island primers. A combination of both methylation PCR and methylation-anchor PCR methods can distinguish the methylated maternally derived allele (*i.e.*, the allele amplified in methylation PCR but not in methylation-anchor PCR) from the unmethylated paternally derived allele (*i.e.*, the allele amplified in methylation-anchor PCR but not in methylation PCR).

In yet other embodiments, samples obtained during epidemiologic studies of patients exposed to chemical or radiation mutagens can be subjected to methylation-

PCR and methylation-anchor PCR to diagnose mutations within genomically imprinted regions.

Definitions

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

As used, the term "sample" is used in its broadest sense. On one hand, it is meant to include a specimen or culture; on the other hand, it is meant to include both biological and environmental samples. Biological samples include blood products, such as plasma, serum and the like. Biological samples may be animal, including human, fluid, solid or tissue. These examples are not to be construed as limiting the sample types applicable to the present invention.

As used, the term "postsynthetic modification" refers to any chemical modification of nucleic acid following replication or transcription (*e.g.*, methylation).

As used, the term "methylated DNA" refers to any nucleic acid sequences where one or more of the nucleotide residues have been covalently modified by the addition of a "methyl group" ($-CH_3$). In certain embodiments, "methylation" involves the enzymatic addition of methyl groups to the C5 position of cytosine or the N6 position of adenosine residues through the action of DNA methyltransferases or DNA methylases.

As used, the term "CpG islands" refers to unmethylated regions within DNA sequences that contain high concentrations of cytosine residues that are directly 5' of a guanine residue. Although a majority of these CG pairs are typically methylated in mammalian genomes, they are mostly unmethylated in CpG islands.

As used, the term "nucleic acid sequence" refers to an oligonucleotide, nucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand.

"Nucleosides" are composed of a purine or pyrimidine base covalently linked to ribose or deoxyribose sugars. "Nucleotides" are made up of a nucleoside with the

addition of up to three phosphate groups linked in series by phosphoanhydride bonds and linked to the sugar by a phosphodiesterase bond. "Adenine," "guanine," "cytosine," "uracil," and "thymine" are the bases. As nucleosides, they are called "adenosine," "guanosine," "cytidine," "uridine," and "thymidine." As nucleotides they are called by their nucleoside name with "mono-," "di-," or "tri-" "phosphate" added, depending on the number of attached phosphate groups.

As used, the term "oligonucleotide" refers to a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and usually more than ten. The exact size will depend on many factors, which in turn depends on the ultimate function or use of the oligonucleotide. The oligonucleotide may be generated in any manner, including chemical synthesis, DNA replication, amplification, reverse transcription, or a combination thereof.

Because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends.

As used, the term "eukaryote" refers to organisms distinguishable from "prokaryotes." It is intended that the term encompass all organisms with cells that exhibit the usual characteristics of eukaryotes, such as the presence of a true nucleus bounded by a nuclear membrane, within which lie the chromosomes, the presence of membrane-bound organelles, and other characteristics commonly observed in eukaryotic organisms. Thus, the term includes, but is not limited to such organisms as fungi, protozoa, and animals (*e.g.*, humans).

As used, the term "transcriptional regulation" refers to the control of gene transcription rates by regulatory factors (*e.g.*, transcription factors) or otherwise.

As used, the term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or precursor. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired polypeptide activity or function is retained.

As used, the term "gene expression" refers to the transcription and translation of a gene.

As used, the term "transcription factors" refers to proteins that interact with RNA polymerase and each other to modulate transcription. The proteins act by binding to regulatory sequences on the DNA or to other proteins that are bound to these regulatory sequences.

Transcriptional control signals in eukaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription (Maniatis, T. *et al.*, *Science* 236:1237 [1987]). Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in plant, yeast, insect, and mammalian cells and viruses. Analogous control elements (*i.e.*, promoters) are also found in prokaryotes.

As used, the term "gene product" refers to the mRNA transcript produced from transcription of the gene or the protein translated from this transcript.

As used, the term "housekeeping genes" refers to genes that encode polypeptides or RNAs who have general functions in all cell types (*e.g.*, cytoskeletal proteins, RNA polymerases, and ribosomal proteins).

As used, the term "nuclear" refers to structures or events present within the nucleus of a cell.

As used, the term "ribonucleoprotein" refers to a complex macromolecule containing both RNA and protein.

As used, the term "polypeptide" refers to a sequence of amino acids.

As used, the term "splicing" refers to the process whereby nucleic acids are cut at specific sites and rejoined.

As used, the term "cytogenetic deletion" refers to the loss of one or more nucleotide pairs from a chromosome. "Large deletion" refers to the loss of enough nucleotide pairs to be observable microscopically. Typically this involves the loss of thousands of nucleotide pairs.

As used, the term "standard cytogenetics" refers to the use of high-resolution analysis to systematically characterize chromosomes. Typically, this involves the use of labeled or stained DNA visualized by microscopy.

As used, the term "fluorescence in situ hybridization" ("FISH") refers to a method of detecting and locating specific DNA or RNA sequences in tissues or on chromosomes. In this method, a fluorescently labelled probe is applied to fixed tissue or chromosome preparations. The probe hybridizes to the target sequence and allows detection by use of fluorescent sensing equipment (*e.g.*, fluorescent microscope). FISH is widely used to map sequences in the genome and to study chromosome aberrations.

As used, the term "microsatellite analysis" refers to methods that use microsatellites (*i.e.*, short repeated DNA sequences), to construct chromosomal linkage maps and to map genetic disease genes to their chromosomal loci.

As used, the terms "amplification," "amplify," or "amplifying," are defined as the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction (PCR) or other technologies well known in the are (*e.g.*, Dieffenbach and Dveksler, *PCR Primer, a Laboratory Manual*, Cold Spring Harbor Press, Plainview NY [1995]). These terms are meant to encompass all present and future nucleic acid amplification technology.

As used, the term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis (*e.g.*, U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188 hereby incorporated by reference), that describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a

DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified".

As used herein, the term "polymerase" refers to any polymerase suitable for use in the amplification of nucleic acids of interest. It is intended that the term encompass such DNA polymerases as *Taq* DNA polymerase obtained from *Thermus aquaticus*, although other polymerases, both thermostable and thermolabile are also encompassed by this definition. "Thermostable polymerases" are polymerases that are capable of functioning at relatively high temperatures.

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of ^{32}P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications.

As used herein, the terms "PCR product" and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence. The process of cutting the DNA is referred to as "restriction digestion." The products of a restriction digestion are referred to as "restriction products."

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (*i.e.*, a sequence of nucleotides) related by the base-pairing rules. For example, for the sequence "A-G-T," is complementary to the sequence "T-C-A." Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Also, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions (*e.g.*, primers hybridizing to the DNA to be amplified), as well as detection methods which depend upon binding between nucleic acids.

The term "homology" refers to a degree of identity. There may be partial homology or complete homology. A partially identical sequence is one that is less than 100% identical to another sequence.

The term "hybridization" refers to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementary between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the nucleic acids.

The term "T_m" refers to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the T_m of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\%\ G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (*see e.g.,* Anderson and Young, Quantitative Filter Hybridization, *in Nucleic Acid Hybridization* [1985]). Other references include more sophisticated computations which take structural as well as sequence characteristics into account for the calculation of T_m.

As used, the term "Southern blot" refers to the analysis of DNA on agarose or acrylamide gels to fractionate the DNA according to size, followed by transfer and immobilization of the DNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized DNA is then probed with a labeled oligo-deoxyribonucleotide probe or DNA probe to detect DNA species complementary to the probe used. The DNA may be cleaved with restriction enzymes prior to electrophoresis. Following electrophoresis, the DNA may be partially depurinated and denatured prior to or during transfer to the solid support. Southern blots are a standard tool of molecular biologists (J. Sambrook *et al.* [1989] *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, pp 9.31-9.58).

As used, the term "restriction fragment length polymorphism" ("RFLP") refers to methods that measure DNA polymorphisms resulting from the loss or creation of DNA at a defined site where a restriction enzyme cuts. DNA containing the alternate structure(s) will give different sized DNA fragments upon digestion with the restriction enzyme.

As used, the term "DNA dosage" refers to the method whereby missing or extra chromosomal material is detected by measuring the amount of gene product from an individual suspected of having at least one chromosome abnormality.

As used, the term "DNA methylation" refers to a method that detects the parent-of-origin-specific methylation imprint at loci that show differential methylation

(*i.e.*, loci with differences in their methylation patterns). The technique is based on Southern hybridization and uses restriction enzymes that specifically digest only methylated DNA or unmethylated DNA (*i.e.*, DNA that has not been methylated).

As used, the term "agarose" refers to the linear polysaccharide polymer extracted from agar that is used to prepare gels for electrophoresis of nucleic acids.

As used, the term "dried blood spots" refers to any blood sample that has undergone partial or complete dehydration.

As used, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product that is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method. "Flanking primers" refers to any primers that are to designed to initiate synthesis, in opposite directions, on both sides of a particular nucleotide sequence. "Island primers" refers to primers that are designed to initiate synthesis within a CpG island.

As used, the term "cell culture" refers to any *in vitro* culture of cells. Included within this term are continuous cell lines (*e.g.*, with an immortal phenotype), primary cell cultures, finite cell lines (*e.g.*, non-transformed cells), and any other cell population maintained *in vitro*.

As used, the term "gel electrophoresis" refers to the method of separating nucleic acids by molecular size by applying an electric field to samples that pulls them through a gel matrix. Larger molecules move more slowly through the matrix.

"Denaturing" refers to the presence of a detergent in the gel that normalizes the charges on proteins and disrupts non-covalent interactions in molecules. "Non-denaturing" or "native" refers to gel electrophoresis conducted in the absence of denaturing detergents.

As used, the term "ethidium bromide" refers to a intercalating compound that is used to stain and visualize DNA bands after agarose gel electrophoresis. The presence of DNA in the gel is detected using ultraviolet light and observing for fluorescing bands. Ethidium bromide fluoresces under ultraviolet light.

As used, the term "radionucleotide" refers to any nucleotide that contains one or more radioactive isotopes covalently attached to the molecule used in an enzymatic reaction.

As used, the term "overhang" or "staggered end" refers to the single-stranded portion of a double stranded DNA molecule remaining after the digestion with a restriction enzyme (*e.g.*, the overhang created in Methylation-anchor PCR that hybridizes to anchor primers).

As used, the term "anchor primer" refers to a primer that hybridizes to the overhang created by a restriction enzyme digestion.

As used, the term "ligation" refers to the process whereby a phosphodiesterase bond is formed between two DNA fragments. The reaction is usually mediated by the enzyme ligase.

As used, the terms "anneal," "renature," or "reassociate," refer to the formation of double stranded nucleic acid through complementary base pairing between separated strands. Annealing can occur between completely complementary strands as well as partially complementary strands and refers to the interaction between any types of nucleic acid, DNA/DNA, RNA/RNA, or RNA/DNA.

As used, the term "genetic abnormality" refers to any deviation in the DNA composition or structure of a chromosome from wild-type. This includes, but is not limited to, deletions, mutations, duplications, rearrangements, covalent modifications, uniparental disomy, and altered chromatin structure.

As used, the term "genomic imprinting" refers to any process in which epigenetic information is introduced into chromosomes and is stably replicated with the chromosome when it divides.

As used, the term "imprinting region" refers to a portion of a chromosome that possesses genomically imprinted genes. The portion includes the sequence of the genes themselves and the sequences important for the function and regulation of these genes.

As used, the term "genome" refers to the total DNA in a single cell.

As used, the term "allele" refers to a variant form of a given gene. "Active allele" refers to a copy of the gene that is, or is capable of, being actively transcribed and translated. "Suppressed allele" refers to a copy of the gene that is repressed and not capable of activation through the normal regulatory mechanisms responsible for inducing transcription.

As used, the term "15q11-q13" refers to a region of human chromosome 15 located on the long (q) arm, 3' of the centromere.

As used, the term "genomically imprinted genes" refers to genes whose expression depends on whether they are carried on the maternally or paternally derived chromosome.

As used, the terms "maternally/paternally contributed chromosomes" refer to the chromosomes inherited from the female and male parents respectively.

As used, the term "uniparental disomy" refers to the condition when one or the other parental genome are present in two copies, with the other absent.

As used, the term "duplication" refers to a type of chromosome aberration in which part of the chromosome is duplicated. This can include, but is not limited to, uniparental disomy, the complete duplication of one of the parental chromosomes.

As used, the term "mutation" refers to the process whereby changes occur in the quantity or structure of the genetic material of an organism. These changes include, but are not limited to, point mutations, deletions, duplications, rearrangements, and inversions.

As used, the term "chromosome rearrangement" refers to the structural changes in the chromosome whereby breakage and rejoining results in portions of the chromosome being moved to new locations within the same or other chromosomes.

As used, the term "Prader-Willi syndrome" refers to the syndrome characterized by neonatal hypotonia, hypogonadism, mild to moderate mental retardation, learning disabilities, obsessive-compulsive disorder, short stature, and morbid obesity due to hyperphagia beyond infancy and associated with genetic aberrations of the paternal chromosome region 15q11-q13.

As used, the term "Angelman syndrome" refers to the syndrome characterized by severe mental retardation, lack of speech, epilepsy, easily evoked laughter, hyperactivity, and ataxic gait and associated with genetic aberrations of the maternal chromosome region 15q11-q13.

As used, the term "inherit" refers to the process whereby an offspring acquires the characteristics of its parents. These characteristics include, but are not limited to, DNA and genomic imprinting.

As used, the term "loci" or "locus" refers to the positions of genes or any characterized DNA sequences on chromosomes.

As used, the term "mapping" refers to the process of determining the position of loci on a chromosome. The methods of mapping include, but are not limited to, somatic cell hybrids, chromosome sorting, in situ hybridization, linkage analysis, dosage analysis, and comparative mapping.

As used, the term "genotypes" refers to the actual genetic make-up of an organisms, while "phenotype" refers to physical traits displayed by an individual.

As used, the term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that

naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

As used, the term "methylation PCR" refers to a method whereby DNA is first digested with methylation-sensitive restriction enzyme and then amplified. It is intended that all amplification methods be encompassed within this definition, including, but not limited to, PCR.

As used, the term "methylation-anchor PCR" refers to a method of this invention whereby DNA is digested with methylation-sensitive restriction enzyme, ligated to anchor primers, and amplified. It is intended that all amplification methods be encompassed within this definition, including, but not limited to, PCR.

As used, the term "mutagen" refers to any chemical or physical agent capable of causing a change in DNA such that the DNA sequence is altered and a mutation produced. "Chemical mutagen" refers to any chemical that acts as a mutagen. "Radiation mutagen" refers to any ionizing radiation or radioactive emitter that acts as a mutagens.

As used, the term "imprinting mutations" refers to mutations in the genomic imprinting process that lead to abnormal DNA methylation and imprinted gene expression.

As used, the term "sufficiently intact genomic DNA" refers to DNA that contains few enough genetic abnormalities caused by the process of isolation, handling, and storage to allow accuracy of experimental analysis.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: M (Molar); mM (millimolar); μM (micromolar); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); pmol (picomoles); g (grams); mg (milligrams); μg (micrograms); ng (nanograms); l or L (liters); ml (milliliters); μl

(microliters); cm (centimeters); mm (millimeters); μm (micrometers); nm (nanometers); μCi (microcurie); °C (degrees Centigrade); γ-^{32}P ATP (ATP labeled with phosphorous-32 at the gamma phosphate); NaCl (sodium chloride); SDS (sodium dodecyl sulfate); TE (10 mM Tris/ 1 mM EDTA); EDTA (ethylenediaminetetraacetic acid); CO_2 (carbon dioxide); ATP (adenosine 5'-triphosphate); SSC (saline-sodium citrate); $MgCl_2$ (magnesium chloride); KCl (potassium chloride); dNTP (deoxyribonucloside 5'-triphosphate); PCR (polymerase chain reaction); RT-PCR (reverse transcriptase PCR); cDNA (complimentary DNA); kb (kilobase); SNRPN (small nuclear ribonucleoprotein particle associated polypeptide SmN); FISH (fluorescence in situ hybridization); MP (Methylation PCR); MAP (Methylation-anchor PCR); Intergen (Intergen, Inc., Purchase, NY); U.S. Biochemical (U.S. Biochemical Corp., Cleveland, OH); Scientific Products (McGraw Park, IL); Sigma (Sigma Chemical Co., St. Louis, MO.); Perkin-Elmer (Perkin-Elmer Co., Norwalk, CT); Oncor (Oncor, Inc., Gaithersburg, MD); Schleicher and Schuell (Schleicher and Schuell, Inc., Keene, NH); New England Biolabs (New England Biolabs, Inc., Beverly, MA); and NEN Research Products (New England Nuclear Research Products, Inc., Boston, MA)

EXAMPLE 1

Preparation of Genomic DNA

In this Example, DNA was extracted from human blood samples, cell culture, or dried human blood spots. Human blood samples were obtained from D.J. Driscoll, University of Florida, Gainesville (Angelman syndrome samples) and S. Schwartz, Case Western Reserve University (Prader-Willi syndrome samples) or were in the possession of the inventors Robert Nicholls and Shinji Saitoh. Samples were obtained after approval from the institutional human subjects review board and informed consent of the individuals or parents.

DNA Extraction from Blood Samples

Genomic DNA was extracted from peripheral blood leukocytes using standard procedures as described by Ausubel (Ausubel *et al.*, [1989] *Current Protocols in Molecular Biology*, John Wiley & Sons, New York NY, pp 2.2.1-2.2.2).

DNA Extraction from Cultured Cells

Lymphoblastoid culture cells derived from AS or PWS patients were maintained in RPMI 1640 medium (80%, fetal bovine serum, 20%, antibiotic free), pH 7.2 in suspension. Cells were kept at 37 °C in a 5% CO_2, 95% air mixture. Genomic DNA was extracted from the cells using standard procedures as described by Ausubel (Ausubel *et al.*, *supra*).

DNA Extraction from Dried Blood

Genomic DNA was extracted and purified from 5 mm diameter blood spots dried on filter paper discs (Schleicher and Schuell no. 903 filter paper). The blood spot samples were cut into four pieces, combined, and soaked with 15 µl of methanol in a 1.5 ml microcentrifuge tube. The tube was incubated at 20°C for 5 minutes and methanol evaporated by vacuum centrifugation for 10 minutes. Then, 360 µl of 0.15 M NaCl, 0.5% SDS were added, and the mixture was incubated at 37 °C for 1 hour. Next, 40 µl of 10 mg/ml proteinase K was added and the mixture was incubated for 2 hours at 37 °C. The supernatant was removed to another tube and the disc pieces were rinsed twice with 100 µl of 0.15 M NaCl, 0.5% SDS twice. The supernatants were combined, extracted with phenol and chloroform, ethanol precipitated, and resuspended in 50 µl of TE. Ten µl of microextracted DNA was used for restriction enzyme digestion and PCR as described in Examples 4 and 5.

EXAMPLE 2

Confirming Diagnoses

Fluorescence in Situ Hybridization (FISH)

Fluorescence in Situ Hybridization was carried out using standard procedures as described by Ausubel (Ausubel *et al.*, *supra*, pp 14.7.1-14.7.11), with minor modifications as indicated below.

Hybridization and detection with SNRPN probe (Oncor) was carried out according to the manufacturer's instructions. Detection was performed with a detection system for digoxigenin labeled probe (Oncor).

PCR Microsatellite Analyses

Microsatellite markers within 15q11-q13 were analyzed by PCR using standard techniques as described by Weber and May (Weber and May, Am. J. Hum. Mol. Genet. 44: 388 [1989]), with modifications as indicated below. Primer sequences for loci were obtained from the Genome Database (Johns Hopkins University). For each marker, one of the primers was end-labeled for 1 hour at 37°C in a 10 µl volume consisting of 1.2 µM primer, 25 µCi [γ-^{32}P] ATP at 3,000 Ci/mmol (NEN Research Products), 50 mmol Tris-HCl, pH 7.5, 10 mmol $MgCl_2$, 5 mmol dithiothrietol, and 5 units T4-polynucleotide kinase. PCR amplification was performed with 27-30 cycles of 1 minute at 95°C, 30-50 seconds at 56°C, and 2-10 minutes at 72°C, in a final volume of 15-25 µl. Each reaction consisted of 30-50 ng genomic DNA, 200 µM each of dATP, dCTP, dGTP, and dTTP, 0.4 µM unlabeled primer, 0.06 µM labeled primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.17-0.32 units *Taq* polymerase. After amplification, the reaction was mixed with an equal volume of formamide loading buffer, denatured at 95°C for 5 minutes, and chilled on ice; 3-4 µl of each sample was directly loaded onto 5-8% denaturing polyacrylamide gels which were processed and autoradiographed.

RNA Analysis

RNA was extracted from tissue or cell culture using standard procedures as described by Ausubel (Ausubel *et al.*, *supra*, pp 4.1.2-4.2.7).

RNA was analyzed by Northern analysis using standard procedures as described by Ausubel (Ausubel *et al.*, *supra*, pp 4.9.1-4.9.13). Samples were probed with cDNAs generated from exon -1 to exon 1 of SNRPN using 5'-AGAGTGGAGCGGCCGCCGC-3' (SEQ ID NO: 2) and 5'-CAGATTCCTCGCTACTCC-3' (SEQ ID NO: 3) or from exon 2 to exon 8 of SNRPN using 5'-TGGTGGAACAGCAATCATG-3' (SEQ ID NO: 4) and 5'-CTAAGGTCTTGGTGGACGC-3' (SEQ ID NO: 5).

Alternately, RNA was analyzed by RT-PCR using cDNA generated from total RNA and primers designed to amplify SNRPN transcript: forward primer 5'-CCACCAGGCATTAGAGGTCCAC-3' (SEQ ID NO: 6) and reverse primer 5'-GCAGAATGAGGGAACAAAAAGCTC-3' (SEQ ID NO: 7).

Southern Blot

Southern Blots were carried out using standard procedures as described by Ausubel (Ausubel *et al.*, *supra*, pp 2.9.1-2.9.11), with minor modifications as indicated below.

Samples were probed with cDNA generated from the SNRPN gene by RT-PCR using: forward primer 5'-GGATTTCCAGGCTGAACTGAGG-3' (SEQ ID NO: 8) and reverse primer 5'-ACAAGACGCATTGCAGGGGA-3' (SEQ ID NO: 9).

DNA methylation analysis

DNA methylation analysis was conducted as described for Southern blot analysis above, with modifications as described below. Ten micrograms of genomic DNA were digested with *XbaI* + *NotI*, run on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized to probe. The probe was a 0.6 kb *EcoRI-NotI* fragment which contains exon -1 of SNRPN (Glenn *et al.*, Am. J. Hum. Genet. 58: 335 [1996]).

EXAMPLE 3

Restriction Digestions and Ligations

Methylation-Anchor PCR (MAP) assay.

DNA (100-500 ng) was cut to completion with *Xba*I and *Not*I, using conditions recommended by the manufacturer (New England Biolabs), in a 100 µl total reaction volume, heat inactivated (65°C, 20 minutes), and used for "anchor" ligation. Larger quantities of DNA should not be used in this reaction because incomplete digestion may occur, leaving uncut DNA that will frustrate subsequent amplification reactions. A 12.5 µl aliquot of digested DNA was used for the ligation reaction with 1 µl annealed "anchor" oligonucleotides, 1 mM ATP, and 350 units T4 ligase (TAKARA) in a 25 µl reaction volume. Ligation was carried out at 16 °C for 2 hours. One µl of the ligation mixture (2.5 ng DNA) was used as a subsequent PCR template.

The anchor primer consisted of two single-stranded oligonucleotides (designated as "top" and "bottom") annealed together, leaving a single-stranded overhang complementary to the targeted restriction site. Annealing of anchor oligonucleotides was performed as follows: 3 pmol/ml final concentration of each of "anchor top" and "anchor bottom" oligonucleotides were prepared in 2xSSC, 10 mM Tris (pH 8.0), boiled for 10 minutes, then cooled slowly. Oligonucleotides used for "anchor" ligation were (Munroe *et al.*, Genomics 19: 506 [1994]):

(1) Anchor top:
5'-GGCCGAAGGAGAGGACGCTGTCTGTCGAAGGTAAACGGACGAGAGAAGG GAGAG-3' (SEQ ID NO: 10);

(2) Anchor bottom:
5'-CTCTCCCTTCTACCCGGGAAGTTCGTCAACATAGCATTTCTGTCCTCTCCTT C-3' (SEQ ID NO: 11).

The ligation products were then amplified as described in Example 4.

Methylation PCR (MP) assay.

Genomic DNA (100 -200 ng) was digested to completion with *Hind*III and *Hha*I, using conditions recommended by the manufacturer (New England Biolabs), in

100 µl reaction, and heat inactivated. Larger quantities of DNA should not be used in this reaction because incomplete digestion may occur, leaving uncut DNA that will frustrate subsequent amplification reactions. The reaction products were then amplified as describe in Example 4.

EXAMPLE 4

PCR Amplification

Methylation-Anchor PCR

Ligation-mediated, or anchor, PCR was conducted using 1 µl template, 2.0 mM $MgCl_2$, 200 µM each dNTP, 400 µM each primer, and 2.5 units AmpiTaq "Gold" *Taq* polymerase (Perkin Elmer) in a 20 µl reaction volume.

The following primers were used for PCR:

(1) *SNRPN* CpG island: A, 5'-GCATGCTCAGGCGGGGATGTGTGCG-3' (SEQ ID NO: 12);
B, 5'-CGCTCCCCAGGCTGTCTCTTGAGAG-3' (SEQ ID NO: 13);

(2) Control primer set: *IPW* gene primers:
60C (5'-CTGCATGATTTTTTTTCAAAAA-3') (SEQ ID NO: 14) and
60D (5'-ATATAGGGAGGTTCATTGCACA-3') (SEQ ID NO: 15);

(3) Anchor primer (AP): 5'-ACCCGGGAAGTTCGTCAACATAGCATTTCT-3' (SEQ ID NO: 16)

(4) *GAPDH* gene (GA) control primer set that spans a CpG-island containing a *Not*I site:

5'-GGTCACGTGTCGCAGAGGAGC-3' (SEQ ID NO: 17) and
5'-AGCATCACCCGGAGGAGAAATCGG-3' (SEQ ID NO: 18);

were also used as a control for *Not*I digestion and anchor-ligation PCR. In multiplex PCR reactions with anchor primers and *SNRPN* PCR primers, only the second GA primer was used. The anchor top and bottom as well as anchor PCR (AP) primers were designed based on the original sequences.

PCR was conducted as follows: 94°C for 10 minutes, followed by 32 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds, and a final extension of 72°C for 5 minutes.

Methylation PCR

PCR was conducted as follows: 94°C for 10 minutes, followed by 33 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds, and a final extension of 72°C for 5 minutes. Primers 11 (5'-CTCCCTCCAGACGTGCCGGGC-3' (SEQ ID NO: 19)) and 12 (5'-CAGCTCTTTATTCTTCCCTCA-3' (SEQ ID NO: 20)), that amplify a portion of the *DAX-1* gene on the X-chromosome, were used as a control (Nakae *et al.*, J. Clin. Endocrinol. Metab. 81: 3680 [1996]).

EXAMPLE 5

Gel Electrophoreses

All PCR products were visualized by ethidium bromide staining of samples run on 3% agarose gel electrophoresis using standard procedures as described by Sambrook *et al.* (Sambrook *et al.*, (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Plainview NY, pp 6.50-6.59).

Results of methylation-anchor PCR and methylation PCR using human blood samples of various patients are shown in Figures 6 and 7 respectively. In Figure 6, genomic DNA was digested, and ligated to anchor primers (AP) for subsequent PCR as described above in Example 3. PCR was performed with AP (SEQ ID NO: 16) and island primers (SEQ ID NO: 12 and 13), and primers 60C-60D (SEQ ID NO: 14 and 15) (Wevrick *et al.*, Hum. Mol. Genet. 3: 1877 [1994]) as controls in separate reactions. DNA samples were from normal control individuals as well as AS and

PWS deletion (del), uniparental disomy (UPD), biparental inheritance (BPI) or imprinting mutation (IM) patients. In Figure 7, PCR was performed with AP, *GAPDH* (GA) (SEQ ID NO: 17 and 18) and island (A or B) primers, using methods and abbreviations as for Figure 6. In the second picture, genomic DNA was digested, and then used as a PCR template as described above in Example 3. PCR was performed with island primers A and B, and primers 11-12 (*DAX-1* gene) (SEQ ID NO: 19 and 20) as a control.

Results of methylation-anchor PCR and methylation PCR using dried blood spots are shown in Figures 8 and 9 respectively. DNA was extracted from dried blood spots on filter paper and DNA methylation at *SNRPN* analyzed by methylation PCR and methylation-anchor PCR, as described above for Figures 6 and 7. The *GAPDH* (GA) (Wevrick *et al.*, Hum. Mol. Genet. 3: 1877 [1994]) gene control was multiplexed in the methylation-anchor PCR assays as a control for methylation-based diagnosis using dried blood spots.

A summary of the data from these and other experiments is presented in the following table.

Table 1. Summary of Methylation-PCR and Methylation-Anchor PCR diagnosis of Angelman and Prader-Willi syndromes[a].

		Initial studies		New blood samples		Dried blood spots	
		MP	MAP	MP	MAP	MP	MAP
PWS	Deletion	17	1	14	0	4	0
	UPD	4	0	-	-	-	-
	IM	4	0	-	-	-	-
AS	Deletion	0	5	0	19	0	3
	UPD	2	4	-	-	-	-
	IM	3	7	-	-	-	-
Normal		30	30	-	-	4	4

a. The number of samples showing PCR amplification of the *SNRPN* gene using the methylation PCR and methylation-anchor PCR techniques are shown. Control primers (*IPW* or *DAX-1*) or *GAPDH* (flanking a *Not*I site) show positive amplification in all DNA samples in methylation PCR and methylation-anchor PCR. -, not done.

Two of four AS UPD patients and three of seven AS imprinting mutation patients showed faint PCR amplification with island primers A and B in the Methylation-PCR assay (Table 1). One of 39 PWS samples, from an imprinting mutation patient, showed weak amplification of *SNRPN* in the Methylation-Anchor PCR assay. This latter DNA sample was extracted in another laboratory and standard Southern blot analysis previously showed this one PWS sample to be atypically and partially demethylated (Sutcliffe *et al.*, Nat. Genet. 8: 52 [1994]). Therefore, fresh patient samples were obtained from 19 AS deletion blood samples and 14 PWS

samples from blood, and all demonstrated the predicted amplification patterns specific for AS or PWS, respectively, using methylation-anchor PCR and methylation PCR assays (Table 1).

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

CLAIMS

We claim:

1. A method for diagnosing genomic imprinting disorders:

a) providing:

i) a biological sample suspected of containing genomically imprinted DNA;

ii) two or more nucleotide primers that are complementary to a portion of human chromosome 15;

iii) at least one methylation-sensitive restriction enzyme; and

iv) DNA polymerase;

b) isolating said DNA from said biological sample;

c) digesting said DNA with said methylation-sensitive restriction enzyme to create a restriction product;

d) exposing said primers to said restriction product; and

e) amplifying said restriction product to produce amplification products.

2. The method of claim 1 further comprising step f) comparing said amplification products to at least one control sample.

3. The method of claim 1, wherein said genomic imprinting disorder is Angelman syndrome.

4. The method of claim 1, wherein said biological sample is from a patient suspected of having DNA damage.

5. The method of claim 4, wherein said DNA damage is caused by a mutagen selected from the group consisting of chemical and radiation mutagens.

6. The method of claim 1, wherein said amplifying comprises the polymerase chain reaction.

7. The method of claim 1, wherein said polymerase is thermostable.

8. The method of claim 1, wherein said portion of human chromosome 15 comprises the sequence between 15q11-15q13.

9. The method of claim 1, wherein said primers flank the SNRPN gene.

10. The method of claim 9, wherein said primers are selected from the group consisting of SEQ ID NO: 12 and SEQ ID NO: 13.

11. The method of claim 1, wherein said methylation-sensitive restriction enzyme is selected from the group consisting of *NotI* and *HhaI*.

12. The method of claim 1, wherein said DNA is genomic DNA.

13. A composition comprising at least a portion of the nucleic acid sequence complementary to SEQ ID NO: 1, or variants thereof.

14. The composition of claim 13, wherein said portion comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 12 and SEQ ID NO: 13.

15. A method for diagnosing genomic imprinting disorders:

a) providing:

i) a biological sample suspected of containing genomically imprinted DNA;

ii) at least two nucleotide primers;

iii) at least one methylation-sensitive restriction enzyme;

iv) a ligation means;

v) one or more anchor primers; and

vi) DNA polymerase;

b) isolating said DNA from said biological sample;

c) digesting said DNA with said methylation-sensitive restriction enzyme to create a restriction product;

d) ligating said anchor primers to said restriction product using said ligation means;

e) exposing said nucleotide primers to said restriction product; and

f) amplifying said restriction product to produce amplification products.

16. The method of claim 15, further comprising step g) comparing said amplification products to at least one control sample.

17. The method of claim 15, wherein said genomic imprinting disorder is Prader-Willi syndrome.

18. The method of claim 15, wherein said tissue sample is from a patient suspected of having DNA damage.

19. The method of claim 18, wherein said DNA damage is caused by a mutagen selected from the group consisting of chemical and radiation mutagens.

20. The method of claim 15, wherein said amplifying comprises the polymerase chain reaction.

21. The method of claim 15, wherein said polymerase is thermostable.

22. The method of claim 15, wherein said nucleotide primers are complementary to a portion of human chromosome 15.

23. The method of claim 22, wherein said portion comprises the sequence between 15q11-15q13.

24. The method of claim 15, wherein said nucleotide primers flank the SNRPN gene.

25. The method of claim 24, wherein said nucleotide primers are selected from the group consisting of SEQ ID NO: 12 and SEQ ID NO: 13.

26. The method of claim 15, wherein said methylation-sensitive restriction enzyme is selected from the group consisting of *NotI* and *HhaI*.

27. The method of claim 15, wherein said DNA is genomic DNA.

FIGURE 1

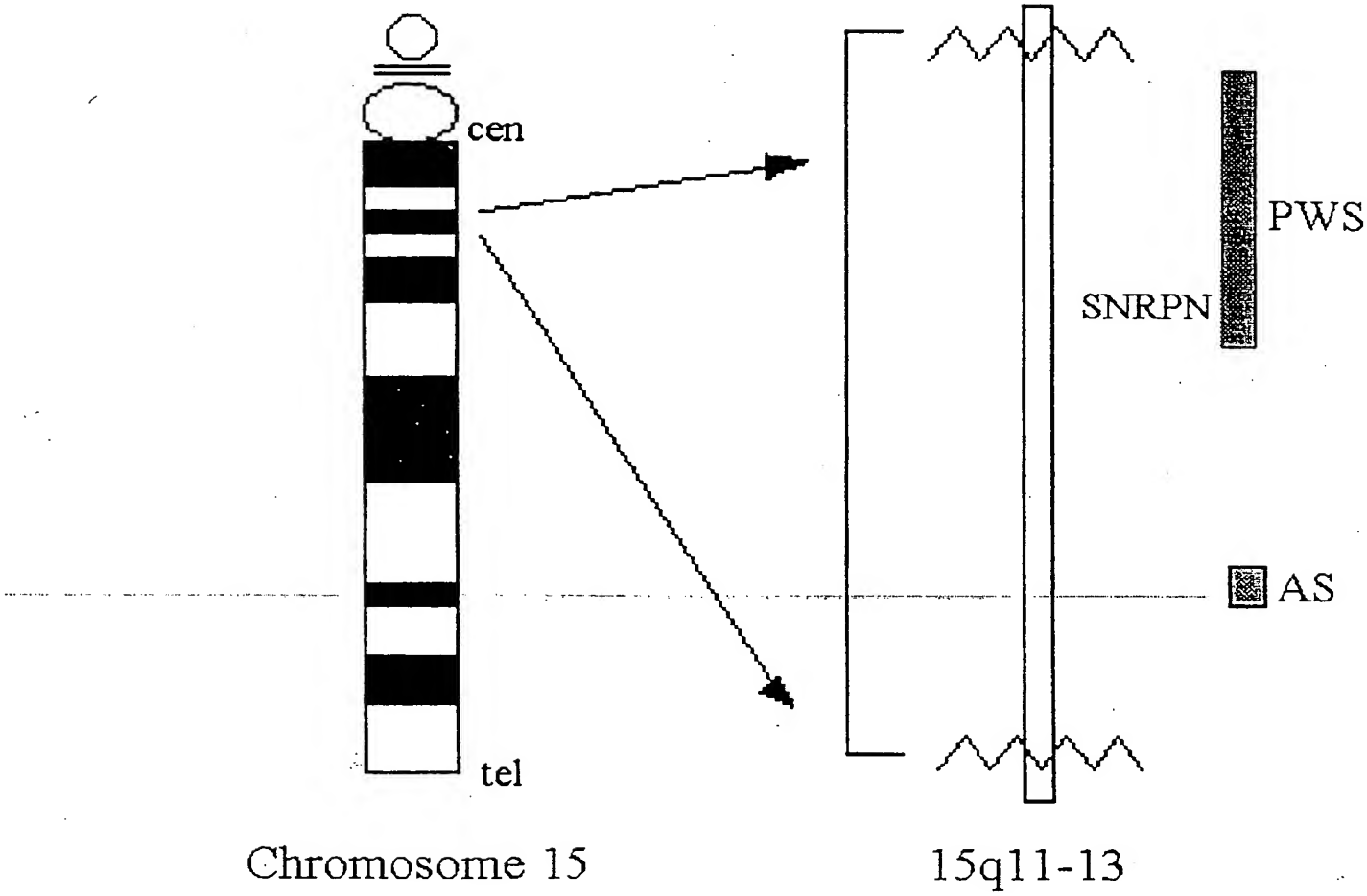

FIGURE 2

ATAGCCAGTG TTTGAGTGAA TGATCTATCC ATTCCGTATA GGAGAGCTAT
AATTATATTG TGATTTGGGG AGATAATTTC ACAATTTACC CCNNAAATGT
AGAAAGTAGA AAAAAGGTAG AAAAAAATNN GCCCAAATTT GAACAATGTA
GCATGCTTTT AGAGTTAGGG ATATTTTCTT TTGGAAGGAT TTCAAGTCTG
GCGTATTTGC ATTGATTGTG GTTATGGCGC ATTTAACNTT TCCAAACCAG
CTTTTTTGTA CCGCACATAG GAAGACCTGA GGGTGAGTGT AAATTAGGAT
TGTTTGGCCA GTGGTGTGTG CAAAAAATTT ATTGTAGACC AAGAATTATT
TCTTTAGTAT TTTACACATT TTAAAAAACA GGTAGACATG TCCATTGATC
CCAGGTTGCT TATGGtTTCT AGAGGCCCCC TCTCATTGCA ACAGTGCTGT
GGGGCCTAGG GGTCCTAGTA GCCCCCTCcc CCCAGGTCAT TCCGGTGAGG
GAGGGAGCTG GGACCCCTGC ACTGCGGCAA ACAAGCACCG CTGGCGGCCG
CAGAGGCAGG CTGGCGCGCA TGCTCAGGCG GGGATGTGTG CGAAGCCTGC
CGCTGCTGCA GCGAGTCTGG CGCAGAGTGG AGCGGCCGCC GGAGATGCCT
GACGCATCTG TCTGAGGAGC GGTCAGTGAC GCGATGGAGC GGGCAAGGTC
AGCTGTGCCG GTGGCTTCTC TCAAGAGACA GCCTGGGGAG CGGCCACTTT
TATTCATCAG ATATTCCAAG TTTTTAGGAC TTGGAGTACT GAATAAACGG
AATTTGGGCC CTAAAGTCCT TTGTTCTGGA GAACCAGATC CGGAATGTTC
AGTGGCTTGC cTGTTGgGTT CccGATGGTA TCTGTCCGCT CGCATTGGGG
CGCGTCCCAT CCGCCCCAAC TGTGGTGTCG CGACAGGTCc TATtGCGGgT
GTCTGCGGTN GGAAGGGCGG TGGTGACTGG GAGCATGCGG GTAACACGTG
GGCAAGGGAC ATGGGTGGAG GTGGGTACAT CAGGGTGATT GCAGTTCCGT
GTGGCGAGGG TACGTGGGGG GACCAGTGCA TAGGGATTTT AGGCGGAGGT
AGGTATATTG GAGTGATTGT GGCGGGGCGA AGGTACGTGA AGTGATCGGT
ATTTAGGGNN NGTTGAGCGC AGGTAGGTGT ATAATAGTGA CTGCGTGGTG
GAGCAGGGTA CGTGGGGCGA CTGGGGGGGA ATTCGTCGCA GTGACCGAGG
CGAGGAGGCT ATGGCAGTGG ACCAGGGGGA TGAGTCGGTG TGACAGTGGT
GGGGGTTGTG TTGGTGGTCG CGGCNGGGAG AGTCCGTAGA AGTGGCTGGG
AGGTAGGGGG AAGGCGGCGA CAGTGGGTAT TGGCGGCGGT GGGCATTGGC
AGCGGGTAGG CATGGCGGTG GTGGACTTTG GCGGCGGTAG GCATGGCGGC
GGTGGGCATG GCATGGAGGC GGTGGGCATG GCGGCCGCGG GCTGTCGCTG
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TCAGAAGGGG CAGTAGCACA GTCGCAGCCT TAATTTCTGT GAC

FIGURE 3

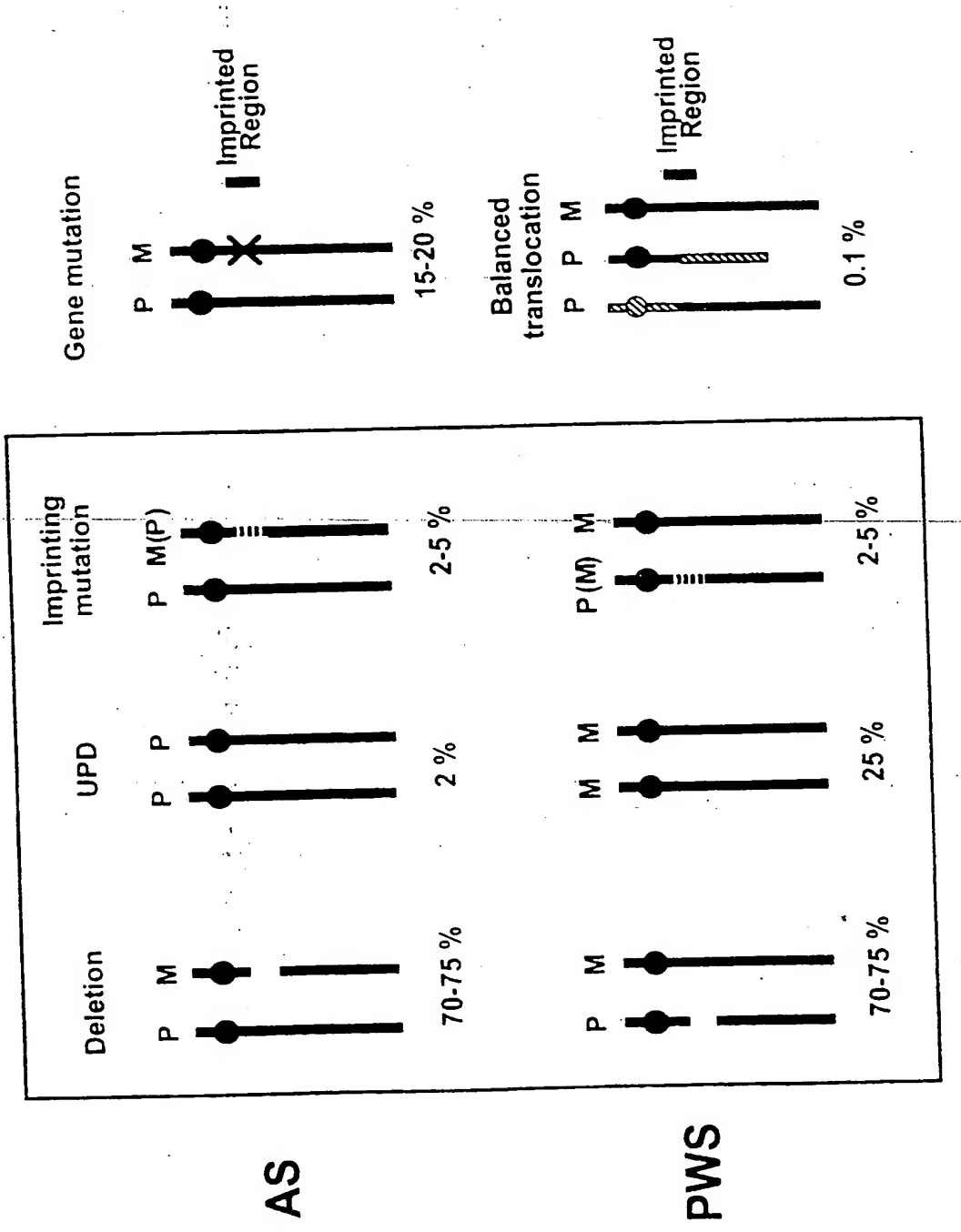


FIGURE 4

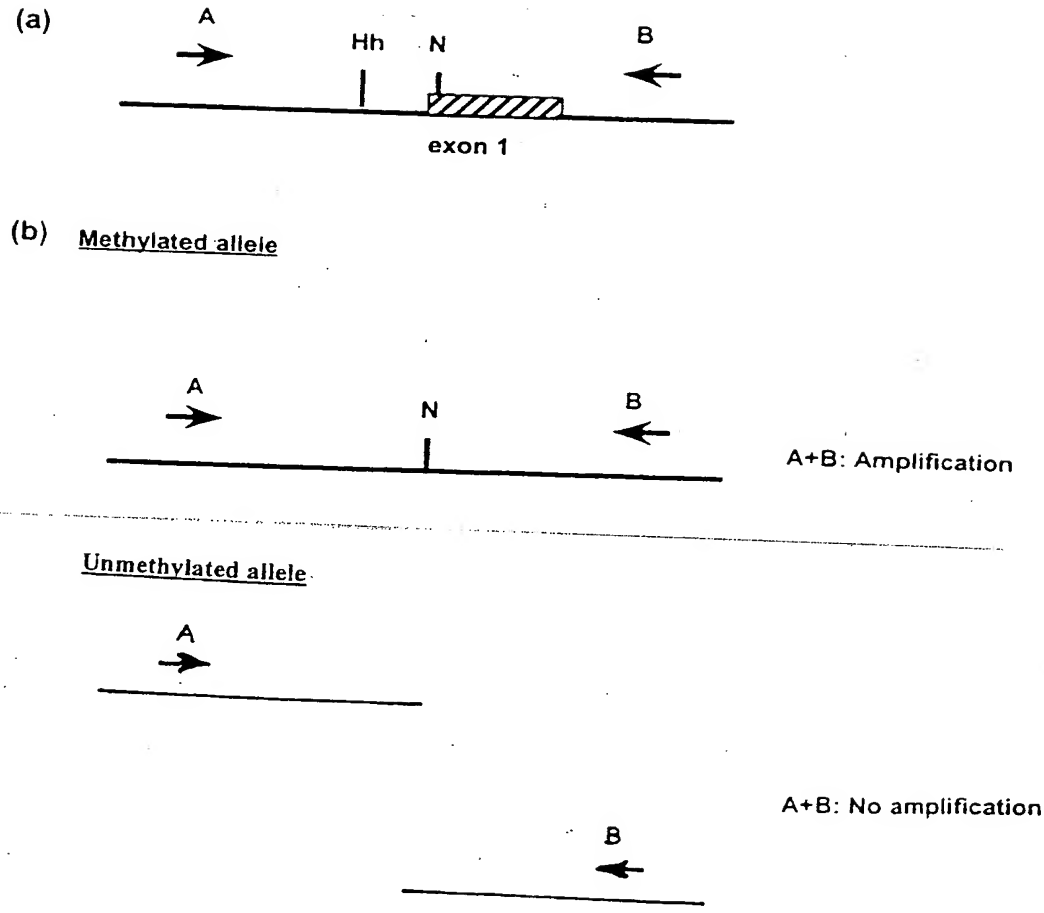

(a)
A
Hh
N
B
exon 1
(b)
Methylated allele
A
N
B
A+B: Amplification
Unmethylated allele
A
A+B: No amplification
B

FIGURE 5

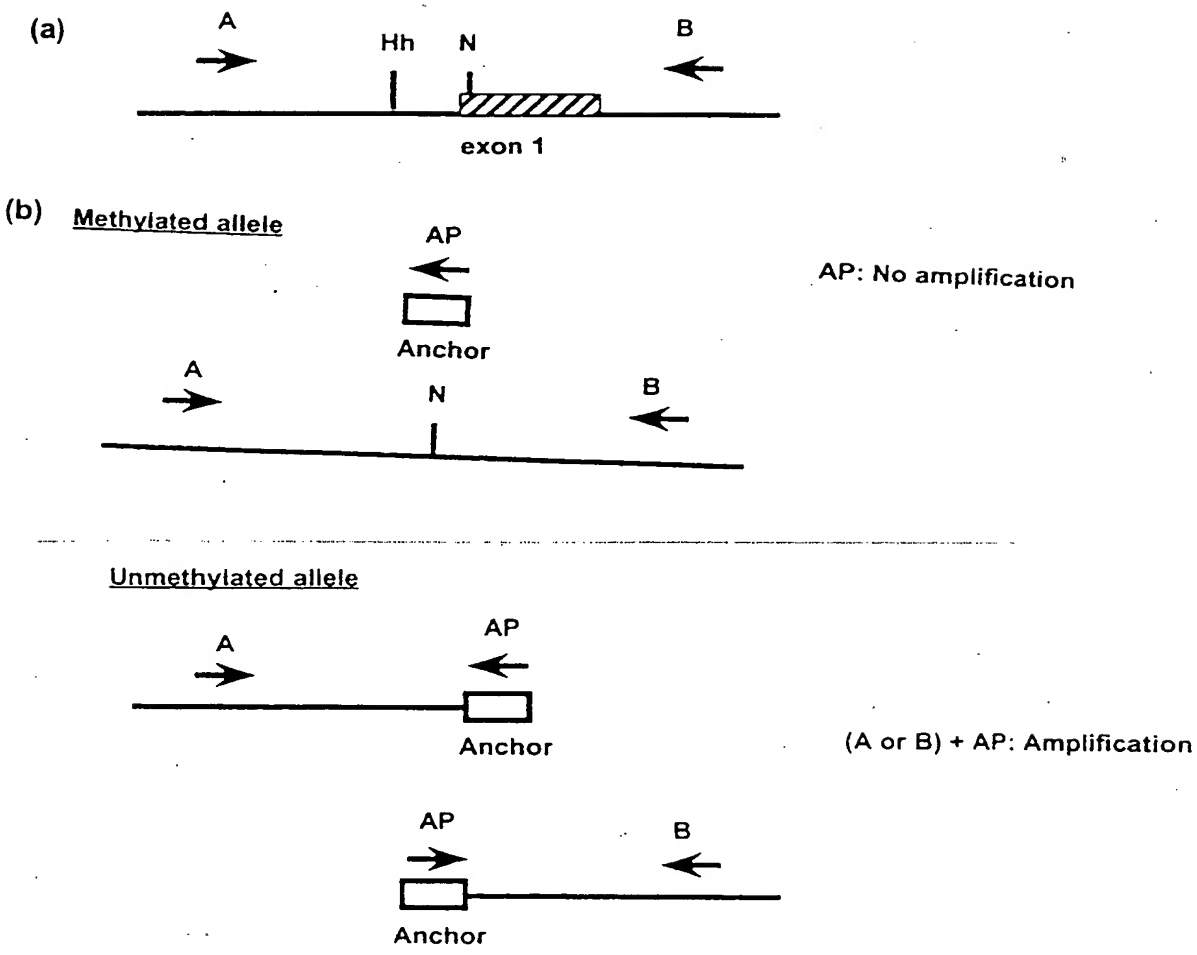


FIGURE 6

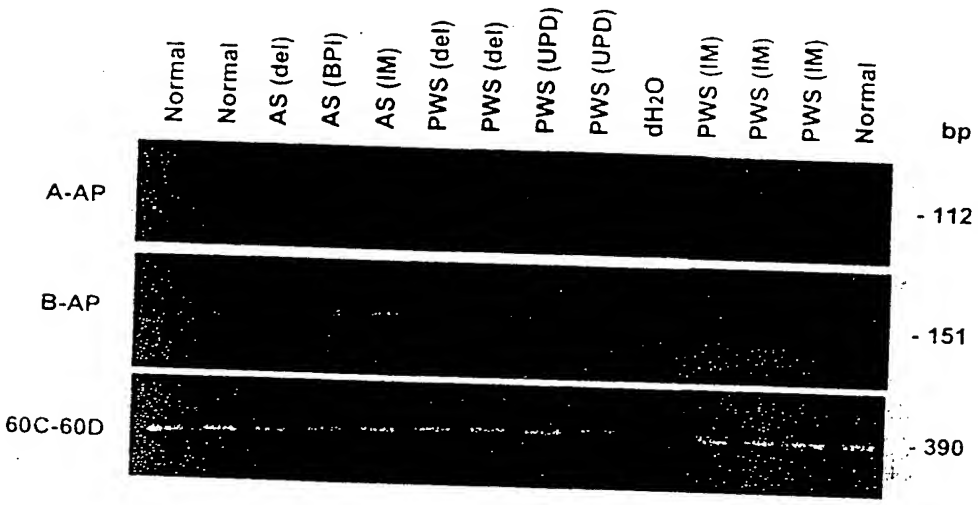


FIGURE 7

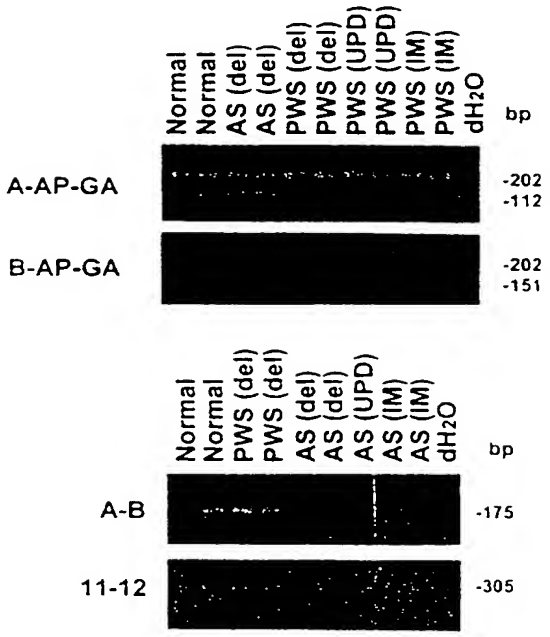


FIGURE 8

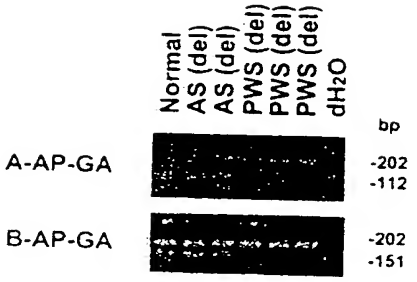
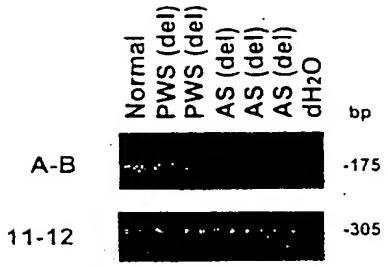

Normal
AS (del)
AS (del)
PWS (del)
PWS (del)
PWS (del)
dH2O
bp
A-AP-GA
-202
-112
B-AP-GA
-202
-151

FIGURE 9



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13818

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68; C12P 19/34; C07H 21/00, 21/04;
US CL : 435/6, 91.2, 91.52; 536/25.3, 24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2, 91.52; 536/25.3, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS, MEDLINE, BIOSIS
search terms: amplification, primers, ligation, small nuclear ribonucleoprotein-associated polypeptide N gene

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	SUTCLIFFE et al. Deletions of a differentially methylated CpG island at the SNRPN gene define a putative imprinting control region. Nature Genetics. September 1994, Vol. 8, pages 52-58, see entire document.	1-12 ----- 13-27
Y	WU et al. The ligation amplification reaction (LAR)-amplification of specific DNA sequences using sequential rounds of template-dependent ligation. Genomics. Vol. 4, pages 560-560, see entire document.	15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
05 OCTOBER 1998

Date of mailing of the international search report
30 OCT 1998

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